

Herpes Simplex Virus Targeting to the EGF Receptor by a gD-Specific Soluble Bridging Molecule

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Herpes simplex virus 1 (HSV-1) enters cells via initial binding of envelope glycoproteins (g) C and B to cell-surface glycosaminoglycans (GAGs) and subsequent membrane fusion involving envelope gD, gB, and gH/gL. Current insights suggest that the fusion process is initiated by interaction of gD with a cognate cellular receptor, such as the widely distributed cell adhesion molecule nectin-1. To redirect the tropism of HSV-1, we have generated a soluble adapter protein (P-V528LH) comprising the gD-binding variable domain of nectin-1 fused to a single-chain antibody (528LH) recognizing the EGF receptor. The adapter molecule enabled HSV-1 entry into naturally nonpermissive CHO cells expressing the human EGF receptor, but not into CHO cells lacking the receptor, and entry was not observed when the antibody portion of the adapter was replaced with an antibody of different specificity. Adapter-mediated entry increased with the viral dose and was nearly as efficient as direct viral entry into nectin-1-bearing CHO cells. Entry depended on viral gD and was diminished in the absence of cellular GAGs. These experiments represent the first demonstration that a soluble molecule can direct HSV infection via a new receptor, supporting the possible utility of this approach for HSV retargeting.

Key Words: gene therapy, herpesvirus, bispecific bridging molecule, targeting, glycoprotein D, nectin-1

INTRODUCTION

The application of viral vectors to the treatment of disease by gene therapy often requires infection limited to a specific cell type in which expression of the therapeutic gene is most effective. Viral tropism is principally determined by the ability of viral surface molecules to recognize and interact with cognate cellular receptors. Many viruses have a broad host range and thus retargeting of vectors to novel receptors is a critical goal in vector development. Enveloped viruses present one or more glycoproteins on their surface that mediate initial virus binding to the cell (attachment) and enable envelope fusion with the cell membrane, resulting in virus entry [1]. Herpes simplex virus (HSV) uses multiple glycoproteins to mediate the attachment and entry processes [2,3]. Attachment involves the binding of at least two envelope glycoproteins, gB and gC, to widely distributed cell-surface glycosaminoglycans (GAGs) [4]. Subsequent initiation of the fusion process requires specific cellular receptors for viral

gD [2]. Thus, HSV tropism is determined by the presence of both attachment and entry receptors on the cell.

Three different receptors for HSV-1 gD have been identified: (i) herpesvirus entry mediator A (HveA or HVEM), (ii) nectin-1 or herpesvirus entry mediator C (HveC; also known as poliovirus receptor-related protein 1), and (iii) heparan sulfate (HS) modified by the activity of 3-O-sulfotransferase-3 (3-OST-3). HveA is a member of the TNF- α receptor family with an expression pattern that is largely restricted to cells of lymphoid origin [5]. 3-OST-3-modified HS functions as an entry receptor exclusively for HSV-1, but its cell distribution and relative significance for HSV infection have not been extensively addressed [4]. Nectin-1 is a cell adhesion molecule belonging to the immunoglobulin (Ig) superfamily [6–8]. Nectin-1 is expressed in a wide variety of human tissues and probably functions as the major HSV-1 entry receptor in the central nervous system [7].

Like other Ig superfamily proteins, nectin-1 is a type I transmembrane receptor with an ectodomain consisting

of an amino-terminal variable (V) domain and two constant (C2-like) domains [7,8]. Evidence from several laboratories has demonstrated that the V domain is necessary and sufficient for gD binding [9–11]. Moreover, the V domain expressed as a fusion with homologous or heterologous transmembrane sequences is sufficient to render receptor-deficient cells susceptible to HSV infection [9].

Along with gB and gH/gL, gD is essential for HSV infection of cultured cells [12–14]. Although gD-deficient mutant viruses are capable of cell attachment and wild-type virus can bind to cells that lack gD receptors [5,12,15], fusion does not occur unless gD interacts with a cognate receptor. Antibody and genetic studies, supported by crystallographic results, have identified the N-terminus of gD as a critical region for binding and virus entry via HveA [16,17]. In addition, it has been shown that this region can accommodate a foreign ligand to expand the HSV-1 host range [18]. In contrast, binding regions exclusive for nectin-1 have not been unequivocally identified and attempts to separate genetically the nectin-1-binding and fusion-triggering functions of gD have met with limited success. Thus, redefining the viral host range by genetic manipulation of gD remains a challenging strategy for HSV retargeting.

HSV offers several advantages over other viruses as a gene-transfer vector, including its large capacity to carry foreign genes and high infectivity independent of cell division [19]. However, the broad host range of the virus limits the target specificity of HSV-based therapies so that procedures to restrict the natural tropism of the virus are desirable. Foreign ligands engineered into gC and gD have been demonstrated to extend tropism to HSV-resistant cells expressing the cognate receptors [18,20] or enhance virion binding to permissive cells [21,22], but these manipulations did not restrict the natural tropism of the virus for the wide variety of cells displaying nectin-1. It has been shown for certain enveloped viruses that cognate soluble receptors can enable virus entry into receptor-deficient cells while inhibiting entry into receptor-bearing cells [23,24]. Furthermore, bispecific soluble proteins (“adapters”) assembled as fusions of virus receptors and ligands for unrelated cell surface receptors can reportedly expand viral tropism to naturally resistant cells [25–30] while potentially inhibiting virus interaction with naturally susceptible cells. These findings suggested the possibility that soluble molecules composed of the gD-binding V domain of nectin-1 and a ligand for a cell-surface receptor of choice could render the HSV entry process insensitive to cell-surface nectin-1 but dependent on the targeted receptor.

In this report, we demonstrate that an adapter protein consisting of the nectin-1 V domain fused to an epidermal growth factor receptor (EGFR)-specific single-chain antibody (scFv) enables HSV entry into HSV-resistant, gD-receptor-deficient cells expressing the

EGFR. Entry was dependent on interaction of the adapter molecule with viral gD and cellular EGFR, as shown using specific antagonists of these interactions. Adapter-mediated entry was nearly as efficient as entry via cell-surface nectin-1, reaching approximately 65% of the cells at a multiplicity of infection (m.o.i.) of 3 compared to 75% infection of nectin-1-bearing CHO cells in the absence of adapter, and was diminished by measures limiting virus binding to cellular GAGs. These results are the first demonstration that the soluble adapter approach can be used to target HSV infection to a novel receptor.

RESULTS

Expression and Purification of Soluble Recombinant Protein

Fig. 1A outlines the structure of nectin-1; the soluble, gD/EGFR-bridging adapter P-V528LH; and the control adapter P-VMuc1LH. A DNA fragment encoding the V domain of nectin-1 (residues 1–114 of the mature protein) was placed behind the signal-peptide sequence of bacterial expression vector pRA, followed by a spacer sequence (S1) and a cassette encoding either the light- and heavy-chain variable domains (V_L and V_H) of EGFR-specific monoclonal antibody 528 (mAb-528) [31,32] separated by a second spacer (S2) or a cassette encoding a mucin-1-reactive scFv (unpublished data), preceding c-Myc and His₆ tags and a stop codon. We isolated bacterially produced adapter proteins from the intracellular insoluble (ICIS) fraction by solubilization and passage over a Ni²⁺-affinity column followed by refolding *in vitro* [33]. We analyzed the proteins by denaturing SDS-PAGE and Western blotting using an anti-His-tag antibody (Fig. 1B, left). P-VMuc1LH and P-V528LH migrated at approximately 43 kDa, in agreement with the predicted molecular sizes of these proteins.

We obtained soluble, truncated gD by transfection of a mammalian expression construct containing the coding region for the gD signal peptide and the first 284 amino acids of the mature protein, followed by sequences specifying a c-Myc tag and a C-terminal His₆ tag. We collected media from transfected 293T cells and purified the His₆-tagged protein over a Ni²⁺ column. Soluble gD₂₈₄ was detected on immunoblots at the expected size of 37.5 kDa by both His₆- and HSV-1 gD-specific antibodies (Fig. 1B and data not shown).

We obtained soluble nectin-1 preparations used in this study by expression of suitable constructs in mammalian cells, essentially as described above for soluble gD. sNec1₁₂₃ consisted of the first 123 amino acids of mature nectin-1, representing only the V domain, while sNec1₃₁₇ included in addition the two C domains of nectin-1, representing the ectodomain. sNec1₁₂₃TMC was a mutant version of sNec1₁₂₃ containing three mutations (Q76A, N77A, M85F) that together prevent interaction of

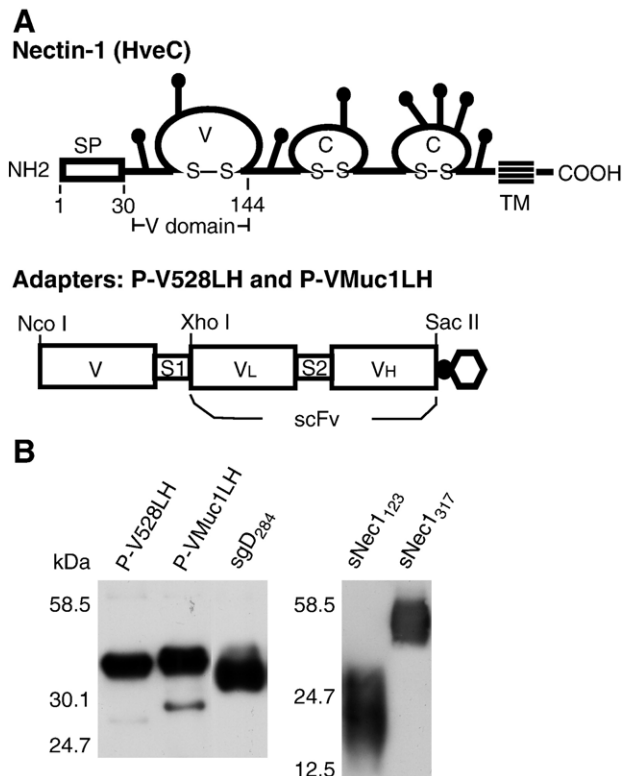


FIG. 1. Bispecific adapter proteins and soluble antagonists. (A) Schematic representations of nectin-1 and genes assembled for adapter expression. Top: SP, signal peptide; V, variable domain of nectin-1; C, constant domains; TM, transmembrane region; S-S, disulfide bonds creating the Ig-like domains of nectin-1; numbers, amino acid positions relative to position 1 of the SP; lollipop, glycosylation sites. Bottom: V, variable domain of nectin-1; S1, Gly₄Ser spacer; scFv, sequences encoding the variable domains of EGFR-specific mAb-528 (528LH) or mucin-1-specific mAb-3C6 (Muc1LH); V_H, heavy chain variable region; V_L, light chain variable region; S2, (Gly₄Ser)₃ spacer. The carboxy-terminal c-Myc and His₆ tags contributed by the bacterial expression vector are represented by a black dot and an open hexagon, respectively. Unique restriction sites used for assembly of the adapter constructs are indicated. (B) Western blot detection of purified recombinant soluble proteins using anti-His-tag antibody.

the V domain with gD [34]. On Western blots, sNec1₁₂₃ was detected by a His₆-specific antibody as a broad band in the 15–30 kDa range, reflecting various degrees of glycosylation (data not shown), while sNec1₃₁₇ migrated at approximately 55 kDa (Fig. 1B, right).

Adapter P-V528LH Mediates EGFR-dependent HSV-1 Infection of CHO Cells

We examined whether the EGFR-specific adapter P-V528LH would allow HSV-1 entry into HSV-resistant CHO-K1 cells and CHO-K1 cells expressing the EGFR. The virus used for these experiments, QOZHG [35], contains expression cassettes for green fluorescent protein (GFP) and bacterial β -galactosidase, enabling rapid detection of intracellular viral gene expression as a signature of virus entry into the cell. Due to deletions

of multiple immediate-early (IE) genes, QOZHG has reduced cytotoxicity, allowing infected cells to survive until scored, and is replication defective, preventing virus amplification and spread.

We inoculated standard CHO-K1 cells and CHO-K1 cells expressing the human EGFR from a stably transfected plasmid (CHO-EGFR cells) with QOZHG virus (m.o.i. of 3) alone or premixed with P-V528LH adapter (30 μ g/ml). While infection of CHO-EGFR cells in the absence of P-V528LH or CHO-K1 cells in the presence of P-V528LH yielded very few GFP-positive cells (Fig. 2A, upper left and lower right), we observed widespread reporter gene expression in CHO-EGFR cells exposed to the combination of virus and P-V528LH (Fig. 2A, upper right). Entry required the EGFR specificity of the antibody portion of P-V528LH, since the structurally identical adapter P-VMuc1LH failed to promote viral-entry-dependent GFP expression in CHO-EGFR cells (Fig. 2A, lower left). By FACS analysis for GFP expression (Table 1) and *o*-nitrophenyl- β -D-galactopyranoside (ONPG) assay for intracellular β -galactosidase activity (Fig. 2B), we showed entry efficiency in the presence of P-V528LH was m.o.i. dependent. In contrast, variation of the adapter concentration over a 10-fold range (6–60 μ g/ml) had mostly minor effects at the multiplicities tested (Fig. 2B), suggesting that adapter availability was not a limiting factor at these levels.

To obtain a measure of the relative efficiency of adapter-assisted entry, we included control infections of CHO-K1 cells expressing human nectin-1 α on their surface (CHO-Nec1 cells) [6] in the experiment shown in Table 1. The results indicated that P-V528LH-assisted QOZHG virus entry into CHO-EGFR cells was nearly as efficient as entry via the natural mechanism of direct virus interaction with cellular nectin-1. We also examined CHO-Nec1 infection after preincubation of the virus with P-V528LH and observed little or no difference compared to infection with virus alone (Table 1). Thus, although the adapter was designed to compete with cellular nectin-1 for binding to viral gD in order to limit the host range of the virus, inhibition of the natural entry mechanism was not observed.

TABLE 1: Percentage of GFP-positive cells after QOZHG infection of CHO-EGFR or CHO-Nec1 cells in the presence or absence of adapter P-V528LH

Cells:	CHO-EGFR		CHO-Nec1	
P-V528LH:	+	-	+	-
m.o.i.				
0.5	ND ^a	ND	26.5	34.6
1	22.9	ND	43.7	46.5
2	36.8	ND	59.4	59.2
3	63.6	ND	75.4	77.2
5	71.9	1.4	84.2	87.9

^a ND, not determined.

Adapter-Mediated HSV-1 Entry Requires Interaction with gD and the EGFR

To verify the mechanism of adapter-mediated entry, we examined the involvement of gD and the nectin-1 portion of the adapter. We analyzed the requirement

for gD by preincubation of the virus/adapter mixture with a pool of gD-specific monoclonal antibodies known to inhibit virus entry into gD-receptor-bearing cells [36]. As shown in Fig. 3A (left), P-V528LH-dependent QOZHG entry into CHO-EGFR cells was strongly reduced by preincubation with 0.5 $\mu\text{g/ml}$ or more of the anti-gD monoclonal antibody (mAb) pool, but not by up to 50 $\mu\text{g/ml}$ anti-c-Myc mAb. Thus, gD appeared to be necessary for adapter-assisted entry. We explored the dependence of this type of entry on the specific interaction of the adapter with gD by (i) competitive-inhibition experiments using soluble forms of gD and the nectin-1 V domain and (ii) preincubation of virus plus P-V528LH with nectin-1-specific antibody CK41, which has been shown to block the V domain gD-binding site [11]. Preincubation of the virus and adapter in the presence of increasing amounts of soluble, truncated gD (sgD₂₈₄) reduced entry measured by ONPG assay compared to preincubation with BSA control protein (Fig. 3A, right). Likewise, preincubation with soluble nectin-1 V domain (sNec₁₁₂₃) or ectodomain (sNec₁₃₁₇) reduced entry in a dose-dependent manner (Fig. 3B, right), while a mutant V domain defective for gD binding (sNec₁₁₂₃TMC) had no effect. Furthermore, entry was decreased by preincubation of the virus and adapter with CK41 antibody compared to c-Myc-specific antibody (Fig. 3B, left). Together, these results confirmed that adapter-mediated entry involved binding of gD to the adapter V domain. We also confirmed that preincubation of CHO-EGFR cells with the EGFR-specific mAb-528 reduced P-V528LH-assisted QOZHG entry in a dose-dependent manner (Fig. 3C), consistent with the suggestion that the adapter interacts with the cells through its mAb-528 domain recognizing the EGFR. As a control, anti-c-Myc antibody had no effect on adapter-dependent entry (Fig. 3C).

Contribution of GAG Binding to Adapter-Mediated Virus Entry

To determine whether virus binding to cellular GAGs is necessary for adapter-mediated entry, we added heparin to the medium prior to infection. As shown in Fig. 4A, increasing concentrations of heparin decreased virus-dependent intracellular reporter gene (*lacZ*) expression to a steady level of approximately 20%, suggesting that virus binding to cellular heparan and perhaps chondroitin or dermatan sulfates enhances the efficiency of adapter-mediated entry.

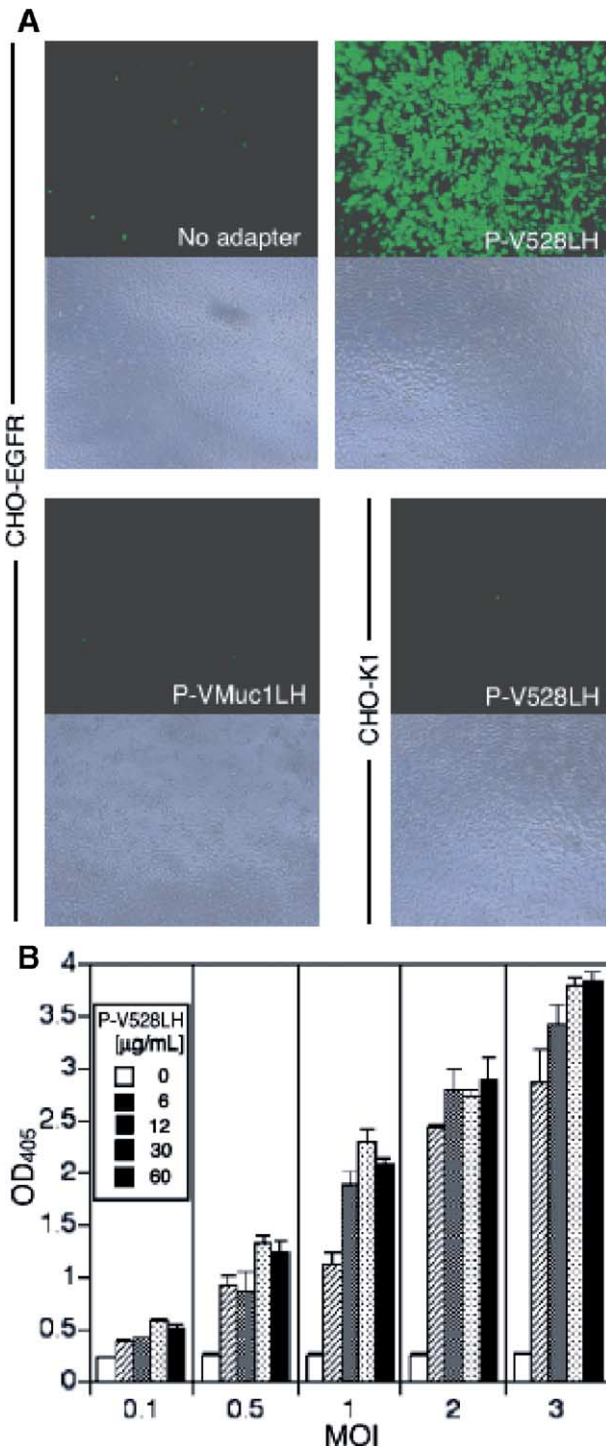


FIG. 2. P-V528LH adapter-mediated HSV-1 infection of CHO-EGFR cells. (A) QOZHG virus (m.o.i. of 3) with or without adapter protein P-V528LH or P-VMuc1LH (30 $\mu\text{g/ml}$) was inoculated onto CHO-EGFR or CHO-K1 cells for 2 h at 37°C. GFP expression was recorded by fluorescence microscopy at 16 h postinfection compared to phase-contrast of the same field. (B) CHO-EGFR cells were infected with premixed QOZHG at m.o.i. of 0.1–3 and P-V528LH at 0–60 $\mu\text{g/ml}$ for 2 h at 37°C. Intracellular *lacZ* expression at 16 h postinfection was determined by ONPG assay.

Second, we tested virus entry on a GAG-deficient CHO cell line that expresses the EGFR. This line, designated CHO-GAG⁻/EGFR, was generated by stable transfection of CHO-derived pgsA-745 cells, which are defective for GAG synthesis [37], with an EGFR expression plasmid. The results in Fig. 4B show widespread GFP reporter gene

expression at m.o.i. of 3 and 10 in GAG-positive CHO-EGFR cells inoculated with virus plus adapter, but limited infection of CHO-GAG⁻/EGFR cells. As estimated by ONPG assay for intracellular *lacZ* gene expression, CHO-GAG⁻/EGFR cells were approximately 10-fold less susceptible to adapter-mediated QOZHG entry than CHO-EGFR

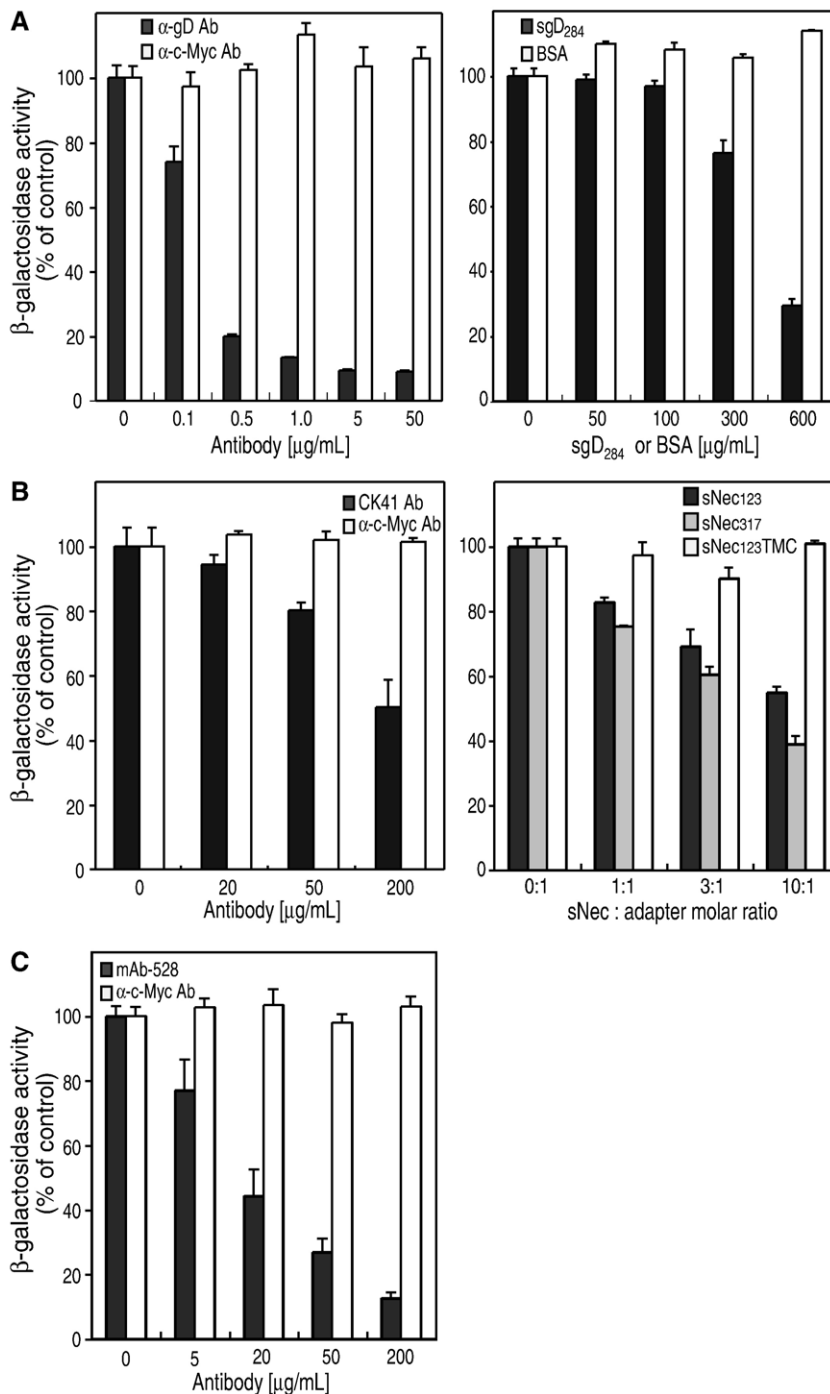
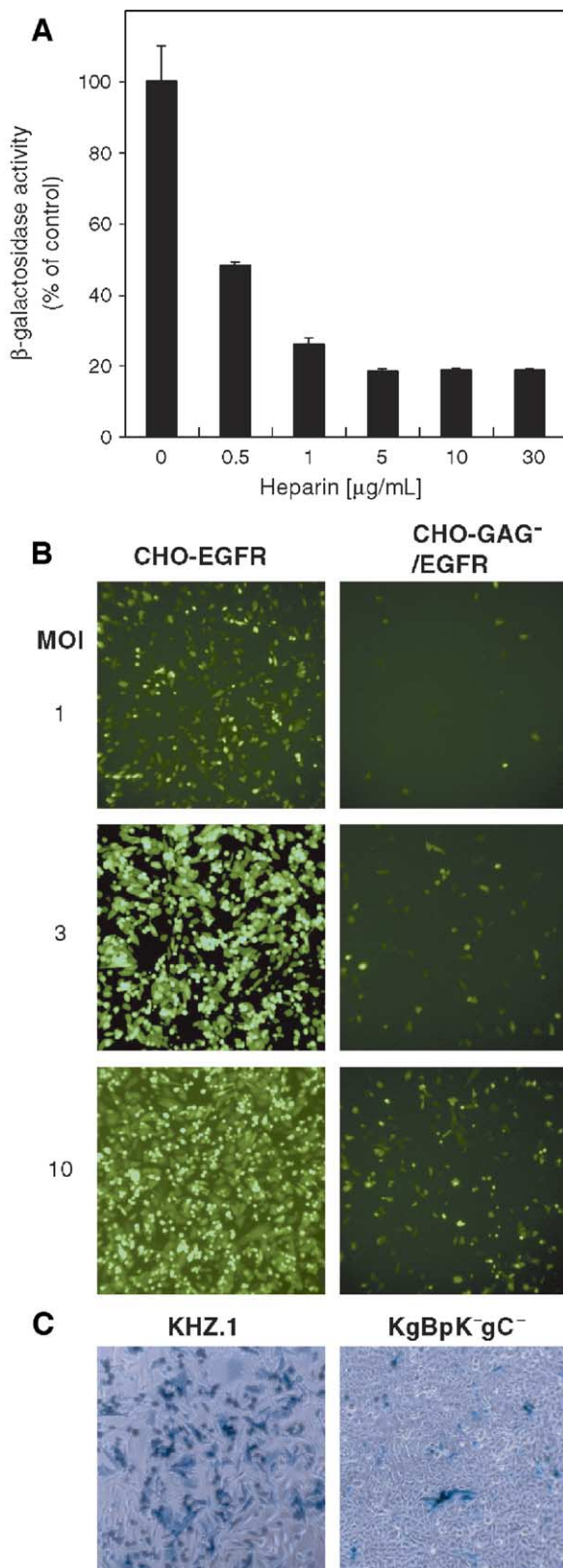


FIG. 3. Involvement of gD, the two adapter domains, and the target receptor in adapter-mediated HSV infection of CHO-EGFR cells. (A) QOZHG virus (m.o.i. of 2) and adapter P-V528LH (12 μg/ml) were incubated with increasing amounts of a mixture of anti-gD mAbs (D3, D7, and D9; Ref. [36]) (left, solid bars), anti-c-Myc mAb 9E10 (left, open bars), soluble gD₂₈₄ (right, solid bars), or BSA (right, open bars) at 4°C for 30 min prior to infection of CHO-EGFR cells. *LacZ* expression was determined at 16 h postinfection by ONPG assay and is plotted as a percentage of the control values (mean = 100%) without antibody (left) or soluble gD or BSA (right). (B) QOZHG (m.o.i. of 2) was incubated with adapter P-V528LH (12 μg/ml) at 4°C for 30 min in the presence of increasing amounts of nectin-1 mAb CK41 (left, solid bars), anti-c-Myc mAb (left, open bars), or (right) soluble nectin-1 V domain (sNec₁₂₃), ectodomain (sNec₃₁₇), or gD-binding-deficient V domain (sNec₁₂₃TMC). CHO-EGFR cells were infected and β-galactosidase activity at 16 h postinfection was determined and plotted as above. (C) CHO-EGFR cells were preincubated with anti-EGFR mAb-528 or anti-c-Myc mAb at 4°C for 1 h, washed three times with PBS, and infected with QOZHG (m.o.i. of 2) preincubated with P-V528LH (12 μg/ml). β-Galactosidase activity was determined and plotted as above.



cells (data not shown). These results demonstrated that cell-surface GAGs, while not essential, promote efficient adapter-mediated infection.

Last, we compared adapter-mediated entry of a replication-competent virus, KHZ.1 [38], and a derivative that is impaired for heparan-sulfate binding due to deletions of gC and the polylysine (pK) tract of gB (KgBpK⁻gC⁻) [39]. As illustrated in Fig. 4C, entry into CHO-EGFR cells was reduced, but not fully abolished by the HS-binding defects of KgBpK⁻gC⁻. Measurement by ONPG assay showed a sevenfold difference between the two viruses (data not shown). Along with confirming the contribution of GAGs to efficient entry, these results also demonstrated that adapter-mediated entry is not limited to the highly defective QOZH mutant virus.

DISCUSSION

Our study demonstrates that a bacterially produced soluble adapter protein, consisting of the variable domain of nectin-1 fused to a single-chain antibody derived from EGFR-specific mAb-528, enables HSV-1 entry into naturally nonpermissive CHO cells expressing the EGFR. This entry mechanism requires viral gD and cellular EGFR and is enhanced by cellular GAGs, presumably functioning as initial virus attachment receptors in the same manner as was demonstrated for HSV infection of susceptible cells [4]. These results show that HSV can be directed to infect cells bearing a targetable receptor without modification of envelope glycoproteins, suggesting that further studies aimed at combining this strategy with methods to eliminate infection by the normal route should provide a suitable means for altering the virus host range.

A number of approaches have previously been explored to alter virus tropism in a manner to achieve cell-specific targeting (reviewed in [27–29]), including pseudotyping [40,41], genetic modification of viral surface proteins [42–45], and the development of bispecific bridging molecules [25,26,30]. Herpesvirus targeting offers unique challenges related to the involvement of multiple envelope glycoproteins in cell recognition and membrane fusion. Some of these glycoproteins (gB, gD) have been shown to function in both processes, and the

FIG. 4. Requirement of virus binding to cellular GAGs in adapter-mediated HSV infection of CHO-EGFR cells. (A) CHO-EGFR cells were preincubated in medium containing increasing amounts of heparin for 1 h at 4°C. QOZH virus (m.o.i. of 2) preincubated with adapter P-V528LH (12 $\mu\text{g/mL}$) was added and the cells were incubated at 37°C for 2 h. *LacZ* expression was determined by ONPG assay at 16 h postinfection and plotted as a percentage of the control value (mean = 100%) without heparin. (B) CHO-EGFR and CHO-GAG⁻/EGFR cells were infected with QOZH (m.o.i. of 1, 3, 10) in the presence of P-V528LH (12 $\mu\text{g/mL}$) for 2 h. GFP expression at 16 h postinfection was recorded under a fluorescence microscope. (C) CHO-EGFR cells were infected for 2 h with KHZ.1 or KgBpK⁻gC⁻ virus (m.o.i. of 1) in the presence of P-V528LH (12 $\mu\text{g/mL}$). *LacZ* expression 16 h after infection was visualized by X-gal staining.

two functions appear to be coupled within gD [2,3]. Moreover, at least three different cell-surface structures are recognized by gD via distinct, but partially overlapping domains, and these domains have not been rigorously separated from the sequences involved in signal transfer to the fusion machinery [2]. Thus, altering the receptor specificity of the different glycoproteins that interact with the cell without impairing the linked fusion process is a delicate operation requiring a detailed understanding of each of the central players.

Our group has previously shown that an HSV-1 mutant presenting erythropoietin on its surface as a chimera with N-terminally truncated, GAG-binding-deficient gC can gain entry via cellular erythropoietin receptors, although by an endocytic pathway resulting in virus degradation [20]. Zhou *et al.* have described an HSV mutant with insertions of IL-13 in both gC and gD, which exhibited IL-13 receptor-dependent progeny virus production [18], but this virus retained the nectin-1-binding domain of gD and thus was still infectious for the wide range of cells expressing cell-surface nectin-1. Compared to these genetic approaches, strategies involving soluble, bispecific adapter proteins offer the benefit that retooling for alternate target cell types is simpler, requiring only replacement of the cell-interacting portion of the adapter molecule instead of construction and isolation of new virus mutants. Our study describes the first functional, bispecific adapter for targeted HSV-1 infection, demonstrating the feasibility of this approach and providing a practical method to examine other receptor/ligand pairs for their ability to effect HSV infection.

Although adapter-mediated infection was highly efficient, adjustments will be required to develop this system into a clinically useful tool. Our laboratory has been the first to identify a set of mutations in gD that abolish gD binding to nectin-1 without impairing HVEM binding or virus entry via HVEM.¹ We have rescued a gD-deficient virus with one of these mutant gD genes and observed normal infection of HVEM-bearing CHO cells without entry into CHO-Nec1 cells (W. A. Shah *et al.*, manuscript in preparation). Since HVEM has a much narrower expression pattern in the body than nectin-1 [5,7], these mutations accomplish a substantial reduction in the virus host range. It is anticipated that this virus can be retargeted using HVEM-based adapters similar in design to the nectin-1-based adapter studied here. Additionally, the observation that a foreign ligand can be functionally inserted into the N-terminal region of gD, thereby disrupting the HVEM-specific binding domain of gD [18], suggests one avenue of converting gD into a highly selective partner for cognate adapters without compro-

missing the essential postattachment role of gD in virus entry. By these procedures, adapters may provide a viable alternative to incorporation of cell-specific ligands directly into gD. Since genetically modified viruses are more stable than two-component systems, gD targeting by genetic modification is preferable over adapter-mediated targeting. However, adapters are likely to accommodate functionally a greater variety of cell-specific ligands than virion gD [28], suggesting that both approaches are worthy of further development.

Two fundamentally different types of herpesviral vectors are under investigation for gene therapeutic applications. Conditionally replicating HSV mutants retaining the full complement of IE genes show promise as oncolytic agents for cancer treatment [27,29]. These vectors derive a measure of target-cell specificity from their replication dependence on complementing activities provided by the host cell, which are highest in rapidly dividing cells. While these vectors may additionally be targeted by modification of the viral envelope, they are less suitable for adapter-mediated targeting because targeted secondary infection by released progeny virus would require the continued presence of adapter. Furthermore, direct cell-to-cell spread would remain uncontrolled without additional provisions. Thus, although an experiment is included in our present report demonstrating that infection by a replicating virus can be targeted by the adapter, the procedure is not initially designed for such vectors. The second type of herpesviral vector is aimed at delivering therapeutic transgenes for prolonged expression in infected cells. We and others have developed replication-incompetent vectors for this purpose that are reduced in cytotoxicity due to deletion of multiple IE genes [46,47]. The QOZHG vector employed in most of our current experiments is an example of such a vector. In this instance, the targeting requirement is limited to the initial infection, "productive infection" defined as the formation of progeny virus is intentionally blocked, and success is indicated by cell-specific expression of a viral reporter gene. Although the mode of entry can affect this endpoint, elucidation of the entry pathway in our model system is of limited value since the HSV entry pathway is believed to vary with both the cell type and the targeted receptor [48,49]. However, such determinations will be important in upcoming stages of this work to identify suitable target receptors and cells for studies *in vivo* aimed at evaluating the clinical promise of the HSV adapter approach.

In summary, we demonstrate that a bispecific adapter protein encompassing an EGFR-specific single-chain antibody fused to the V domain of nectin-1 enables efficient HSV entry via the targeted receptor. Adapter-dependent entry was supported by initial virus interaction with cellular GAGs but was independent of cellular nectin-1. While the biologically active adapter did not restrict the natural tropism of HSV, our study points to alternate designs that, along with a newly developed host-range

¹ Bai, Q., Shah, W. A., Cohen, J. B., Eisenberg, R. J., Cohen, G. H., and Glorioso, J. C. (2001). 26th International Herpesvirus Workshop, Regensburg, Germany, Abstract 2.10, July 28–August 3, 2001.

virus mutant and existing or adapter-based procedures to retarget gC and gB, may accomplish a satisfactory level of target-cell specificity for preclinical evaluation.

MATERIALS AND METHODS

Cell Lines and Viruses

Chinese hamster ovary cells (CHO-K1, ATCC, CCL-61) were maintained in Ham's F-12K medium (Gibco-Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). CHO-Nec1 and CHO-EGFR cells, which are CHO-K1 derivatives constitutively expressing full-length human nectin-1α (HveC) and EGFR, respectively, were kindly provided by Patricia Spear (Northwestern University, IL, USA) and H. Ebina (Tokushima University, Tokushima, Japan). Both cell lines were grown in Ham's F-12K medium supplemented with 10% FBS and 400 µg/ml G418 (Gibco). pgsA-745 (ATCC CRL-2242) is a mutant CHO cell line defective in GAG synthesis [37]. EGFR-expressing pgsA745 cells (designated CHO-GAG⁻/EGFR) were generated by stable transfection of pgsA-745 cells with a human EGFR expression plasmid (kindly provided by Ira Pastan, NIH, Bethesda, MD, USA) and were maintained in Ham's F-12K medium supplemented with 10% FBS and 5 µg/ml blasticidine S (Invitrogen). Replication-defective QOZHG virus, which expresses a single IE gene (ICP0) due to deletions (ICP4, ICP27) and promoter substitutions (ICP22, ICP47), contains an expression cassette for bacterial β-galactosidase at the U₁41 locus and for GFP at U₁54 [35]. QOZHG was grown and titered on ICP4/ICP27-complementing 7b cells [50]. Replication-competent viruses KHZ.1 [38] and KgBpK⁻ gC⁻ [39] were grown and titered on Vero cells.

Plasmids for Expression of Soluble Proteins

Adapter constructs for bacterial expression. A nectin-1 V-domain fragment specifying amino acids 1–114 of the mature protein was obtained by PCR on a human nectin-1α expression plasmid (kindly provided by Yoshimi Takai, Osaka University, Osaka, Japan) using primers that introduced a flanking *NcoI* site upstream and a Gly₄Ser spacer (S1 in Fig. 1A) followed by an *XhoI* site downstream. After digestion with *NcoI* and *XhoI*, the V-domain fragment was inserted between the *NcoI* and the *XhoI* site of bacterial expression vector pRA [31], creating pRA-sNec1₁₁₄. Mouse B-cell hybridoma 528, which secretes a monoclonal antibody against the human EGFR (mAb-528), was kindly provided by Toshio Kudo (Tohoku University, Tohoku, Japan). A previously cloned light-chain variable-domain (V_L) fragment of mAb-528 [31] was modified by introducing a flanking *XhoI* site upstream and a (Gly₄Ser)₃ spacer (S2) followed by an *EagI* site downstream using PCR. A cloned heavy-chain variable-domain fragment (V_H) of mAb-528 was similarly modified to introduce an *EagI* site upstream and a *SacII* site downstream. The modified V_L and V_H fragments digested at the flanking restriction sites were cloned between the *XhoI* and *SacII* site of pRA-sNec1₁₁₄, creating adapter plasmid pRA-V528LH (Fig. 1A). Plasmid pRA-VMuc1LH was derived from pRA-V528LH by replacing the *XhoI*–*SacII* fragment spanning the mAb-528 V_H/V_L region with a corresponding fragment encoding a mucin-1-reactive V_H/V_L region (unpublished data).

Soluble gD and nectin-1 constructs for mammalian expression. A cDNA fragment encoding the signal peptide and N-terminal 284 residues of HSV-1 gD was obtained by PCR amplification on a genomic gD plasmid using primers FgD284 (5'-AAAAGCTAGCCACCATGGGGGGGCTGCCGCCAGG-3') and RgD284 (5'-AAAAGGGCCCCACGGGGTCTC-CAAGAG-3'). The PCR product was digested with *NheI* and *ApaI* (in italic type in the primer sequences) and ligated into *NheI/ApaI*-digested pSecTagA (Invitrogen), which provides a c-Myc tag, six histidine residues, and a TAA stop codon following the *ApaI* site. The resulting recombinant plasmid, designated pgD284-his, was verified by DNA sequencing.

Expression plasmids used in this study for the production of soluble, truncated forms of nectin-1 in mammalian cells (sNec1₁₂₃, mutant sNec1₁₂₃TMC, and sNec1₃₁₇) will be described elsewhere.

Expression and Purification of Soluble Proteins

Bispecific adapters. Previously described procedures were used for expression of pRA-V528LH and pRA-VMuc1LH [33]. Briefly, *Escherichia coli* strain BL21 (DE3) was transformed with either expression vector and grown at 28°C in 2× YT broth with ampicillin (100 µg/ml). To induce expression of the adapter cassettes, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to the cultures and the cells were grown overnight. From 200 ml of culture, bacterial supernatant (BS), intracellular soluble (ICS), and ICIS fractions were obtained as follows. The BS fraction was separated from the cells by centrifugation (2000g, 35 min, 4°C). The cell pellets were resuspended in 80 ml cold phosphate-buffered saline (PBS), ultrasonicated at 150 W for 15 min, and centrifuged at 4500g for 20 min at 4°C. The ICS fraction was recovered as the supernatant. The pellet representing the ICIS fraction was solubilized with 10 ml of 6 M guanidine hydrochloride (Sigma, St. Louis, MO, USA) in PBS (Gu-HCl/PBS) overnight at 4°C. The solubilized ICIS fraction, containing the majority of adapter protein as judged by immunoblotting (data not shown), was passed over a 2-ml TALON Ni²⁺ affinity column (Clontech, Palo Alto, CA, USA). After extensive washing with 6 M Gu-HCl/PBS containing 1 mM imidazole (Sigma), histidine-tagged protein was eluted with 6 M Gu-HCl/PBS containing 250 mM imidazole. For refolding *in vitro*, the purified protein was diluted to a concentration of 1.7 µM and dialyzed for phase-guanidine removal against Gu-HCl/PBS in the presence of L-arginine (Sigma), as previously reported [33]. The material was next dialyzed against PBS (pH 7.9) to remove the L-arginine and centrifuged at 4500g for 20 min at 4°C to remove insoluble material. As a final step, the concentrated samples were sterilized by filtration through a 0.22-µm ultrafiltration membrane (Millipore, Tokyo, Japan).

Soluble derivatives of nectin-1 and gD. 293T cells were transfected with expression vectors for soluble nectin-1 or soluble HSV gD proteins using LipofectAMINE-Plus reagent (Invitrogen) and incubated at 37°C for 3 days. The culture media were collected and enriched for histidine-tagged proteins by passage over a TALON Ni²⁺ affinity column (Clontech) according to the manufacturer's protocol. After concentration by Centricon Plus-20 (Amicon, Bedford, MA, USA) centrifugal filtration, purified protein samples were dialyzed at 4°C against three exchanges of PBS with intervals of 1, 4, and 16 h, divided into 20-µl aliquots, and stored at –80°C. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA).

SDS-PAGE and Western Blotting

Purified protein samples (500 ng) were electrophoresed on 12% polyacrylamide–sodium dodecyl sulfate gels under reducing conditions and blotted onto nitrocellulose membranes (Amersham-Pharmacia, Piscataway, NJ, USA). After transfer, the membranes were treated for 1 h at room temperature with blocking buffer (PBS containing 0.05% Tween 20 and 4% nonfat dry milk) under mild agitation. The membranes were then incubated with peroxidase-conjugated anti-His-tag mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by signal enhancement using the ECL detection system (Amersham-Pharmacia).

HSV-1 Entry Assays

Cells were grown to confluence in 96-well plates (5 × 10⁴ per well) in F-12K/10% FBS medium. QOZHG virus was preincubated with concentrations of adapter protein ranging from 0 to 60 µg/ml in PBS in a total volume of 30 µl for 30 min at 4°C. The culture medium was removed from the cells and the virus/adapter mixture added to the monolayer. After a 2-h incubation at 37°C, the cells were washed once with PBS, overlaid with fresh F-12K/10% FBS medium, and incubated for another 16 h at 37°C. Entry was determined by one or more of the following procedures: (i) visual examination of viral GFP reporter gene expression under a Nikon Diaphot fluorescence microscope (Nikon, Inc., Melville, NY, USA), (ii) flow cytometry to quantitate the number of GFP-positive cells, (iii) visual inspection of fixed cultures incubated with β-galactosidase colorimetric substrate, or (iv) ONPG assay to determine cumulative β-galactosidase levels in infected cultures. For flow cytometry, infected cultures (5 × 10⁵ cells per well) were trypsinized at 16 h postinfection and the cells

collected by centrifugation. After several washes with PBS, the cells were resuspended in 0.5 ml PBS and the percentage of GFP-expressing cells was determined (FACSCalibur; Becton-Dickinson, San Diego, CA, USA). For visualization of β -galactosidase-expressing cells, infected monolayers were fixed with 0.25% glutaraldehyde (Sigma) for 1 min at room temperature, washed with PBS, and incubated with 0.2 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; Sigma). For quantitative determination of β -galactosidase levels, infected cultures were lysed in 150 μ l of 1% NP-40, 1 mM MgCl₂, 50 mM β -mercaptoethanol, and 4 mg/ml β -galactosidase substrate ONPG (Sigma). Samples were incubated at 37°C for 15–20 min until a faint yellow color developed, and absorbance was measured at 405 nm.

Blocking and Competitive-Inhibition Experiments

Interactions with the ectodomain of viral gD. QOZH (m.o.i. of 2) and adapter (12 μ g/ml) were mixed and incubated with various amounts of a pool of gD-specific mAbs (equal amounts of mAbs D3, D7, and D9 [36]) or soluble gD ectodomain (sgD₂₈₄) in PBS at 4°C for 30 min. As controls, anti-c-Myc mAb 9E10 (Santa Cruz Biotechnology) and BSA were substituted for the anti-gD mAb pool and sgD₂₈₄, respectively. Infections and entry assays were performed as described above.

Interactions with the nectin-1 V-domain portion of the adapter. QOZH (m.o.i. of 2) and adapter (12 μ g/ml) were mixed and incubated with various amounts of anti-nectin-1 α antibody CK41 (a gift from Claude Krummenacher, University of Pennsylvania) [11] or truncated nectin-1 protein (sNec1₁₂₃, sNec1₃₁₇, or sNec1₁₂₃TMC) in 30 μ l PBS at 4°C for 30 min. Anti-c-Myc mAb was used as a control for mAb-CK41. Infections and entry assays were performed as above.

Interactions with cellular EGFR. CHO-EGFR cells (5×10^4 cells per well) were incubated for 1 h at 4°C with various amounts of EGFR-specific mAb-528 or control anti-c-Myc mAb in a total volume of 30 μ l adjusted with PBS/1% FBS. The cells were then washed three times with cold PBS and incubated for 2 h at 37°C with QOZH virus (m.o.i. of 2) premixed with adapter protein (12 μ g/ml) for 30 min at 4°C. After replacement of the medium with fresh F12-K/10% FBS, the cells were kept at 37°C for another 16 h and viral entry was examined as above.

Heparin Competition Assay

CHO-EGFR cells (5×10^4 per well) were preincubated with various concentrations of heparin (0, 0.5, 1, 5, 10, 30 μ g/ml) at 4°C for 30 min. QOZH virus (m.o.i. of 2) was mixed with adapter protein (12 μ g/ml), incubated for 30 min at 4°C, and added to the cells. After a 2-h incubation at 37°C, the cells were washed with PBS and cultured in fresh F12-K/10% FBS medium at 37°C for 16 h. Entry assays were performed as described above.

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